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IMPACT OF SIMULTANEOUS STIMULATION OF 5-LIPOXYGENASE AND MYELOPEROXIDASE IN HUMAN NEUTROPHILS

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Abstract

Human neutrophil 5-lipoxygenase (5-LOX) oxidizes arachidonic acid (AA) to 5S-hydro(pero)xy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-H(p)ETE) and leukotriene (LT)A4, which is further converted to the chemoattractant LTB4. These cells contain also the heme enzyme myeloperoxidase (MPO) producing several potent oxidants such as hypochlorous acid (HOCl). Previously, it was shown that MPO-metabolites influence 5-LOX product formation. Here, we addressed the question, whether a simultaneous activation of MPO and 5-LOX in neutrophils results in comparable changes of 5-LOX activity.

Human neutrophils were stimulated with H2O2 or phorbol 12-myristate 13-acetate (PMA) for MPO activation and subsequently treated with calcium ionophore A23187 inducing 5-LOX product formation on endogenous AA. Special attention was drawn to neutrophil vitality, formation of MPO-derived metabolites and redox status. The pre-stimulation with H2O2 resulted in a concentration-dependent increase in the ratio of 5-HETE to the sum of LTB4 + 6-trans-LTB4 in consequence of MPO activation. Thereby no impairment of cell vitality and only a slightly reduction of total glutathione level was observed. An influence of MPO on 5-LOX product formation could be suggested using an MPO inhibitor. In contrast, the pre-stimulation with PMA resulted in different changes of 5-LOX product formation leading to a reduced amount of 5-HETE unaffected by MPO inhibition. Furthermore, impaired cell vitality and diminished redox status was detected after PMA stimulation. Nevertheless, a MPO-induced diminution of LTB4 was obvious. Further work is necessary to define the type of 5-LOX modification and investigate the effect of physiological MPO activators.

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Abbreviations

MVP, 1-methyl-2-vinylpyridinium triflate; 4-ABAH, 4-aminobenzoic acid hydrazide; APF, aminophenyl fluorescein; JC-1, J-aggregate forming cationic dye

Keywords

Leukotriene, HOCl, Inflammation, Glutathione, Hydrogen peroxide

Introduction

The inflammatory response against infectious agents is characterized by a rapid recruitment of neutrophils from peripheral blood to the inflammatory loci driven by chemotactic agents like IL-8, fMet-Leu-Phe and others [Witko-Sarsat *et al.* 2000]. During this movement, neutrophils produce in a 5-lipoxygenase (5-LOX)-dependent reaction the potent autocrine chemotactic molecule leukotriene B_4 (LTB_4). Thereby, 5-LOX, a non-heme-iron dioxygenase, translocates to the nuclear envelope, interacts with 5-LOX activating protein (FLAP) and oxygenates arachidonic acid (AA), which is liberated by the action of cytosolic phospholipase A_2 ($cPLA_2$) [Radmark *et al.* 2015]. The lipoxygenase reaction is initiated by abstraction of a hydrogen atom from C7 of AA by ferric 5-LOX [Rouzer and Samuelsson 1986] generating a radical, which migrates to the C5 position, where it reacts with dioxygen forming a peroxyl radical. Subsequently, the peroxyl anion is formed by interaction with ferrous 5-LOX yielding 5*S*-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HpETE) after protonation [Haeggstrom and Funk 2011]. Human 5-LOX also possesses a secondary leukotriene A_4 (LTA_4) synthase activity [Rouzer *et al.* 1986]. For this purpose a hydrogen atom at the C10 position of 5-HpETE is abstracted, followed by radical migration, rearrangement of double bonds and formation of an epoxide moiety [Maas *et al.* 1982]. Thereafter, unstable LTA_4 is hydrolyzed to leukotriene B_4 (LTB_4) by the action of LTA_4 hydrolase [Newcomer and Gilbert 2010].

During respiratory burst in phagocytosing neutrophils a high proportion of consumed dioxygen (reported values of 28 - 72%) is converted into hypochlorous acid (HOCl) by the heme protein myeloperoxidase (MPO), which is present in azurophilic granules [Klebanoff *et al.* 2013]. Upon reaction with H_2O_2, oxidized MPO can abstract two electrons from chloride generating HOCl [Arnhold and Flemmig 2010]. However, the sole impact of HOCl on microbe killing is a matter of considerable debate [Nauseef 2007; Segal 2005]. When [$^{13}C_6$]-tyrosine labelled *Staphylococcus aureus* are phagocytosed from neutrophils, chlorinated bacterial proteins could be detected only after 5 min leading to microbe killing. But, the majority of chlorinated tyrosine residues, this means 94%, were detected in neutrophil proteins [Chapman *et al.* 2002]. Furthermore, individuals with MPO deficiency, occurring with a relative high prevalence, showed only partially pathogen-dependent problems with severe infections indicating MPO-independent host defense mechanism that compensate in part their lack of MPO [Klebanoff 1970; Kutter 1998; Parry *et al.* 1981]. Therefore, the implication of HOCl in further biological aspects could be suggested. Typical reaction partners of HOCl are thiols, thioether and amino groups. This enables HOCl to oxidize amino acid residues such as cysteine, methionine, tyrosine, tryptophan, and lysine [Pattison and Davies 2001]. Interestingly, with respect to the above mentioned HOCl preference for neutrophil proteins, it could be shown that different granule proteins can be irreversible inactivated by HOCl or the MPO-H_2O_2-Cl^- system [Fu *et al.* 2003; Hirche *et al.* 2005; Shao *et al.* 2005]. Additionally, HOCl can react with amines to produce chloramines or

N-chlorinated derivatives with longer lifetime and membrane permeability [Grisham *et al.* 1984]. Furthermore, HOCl can readily pass through membranes assuming MPO-dependent oxidative modification of cytosolic proteins [Vissers and Winterbourn 1995; Wilkie-Grantham *et al.* 2015].

Both neutrophil enzymes, 5-LOX and MPO, are sequentially activated during migration and in response to pathogen recognition, respectively. Here, the question arises whether HOCl, a potent amino acid-oxidizing agent, could affect the activity or product profile of 5-LOX. Previously, we demonstrated that MPO-derived oxidants exhibited a considerable impact on human recombinant 5-LOX, impairing the epoxidation of 5-HpETE, whereas the hydroperoxidation of arachidonic acid was unaffected [Zschaler *et al.* 2015]. Thereby, HOCl and HOBr, a further prominent hypohalous acid produced from peroxidases, increased the ratio of 5-H(p)ETE to 6-*trans*-LTB_4 in a concentration-dependent manner. Comparable results were obtained with the MPO-H_2O_2-Cl^- system, when glucose oxidase and glucose were applied as a source of H_2O_2. This was necessary due to a strong impairment of 5-LOX activity by H_2O_2. These results implicate, that MPO could terminate the 5-LOX dependent formation of LTB_4, raising the question of whether that could be of importance in human neutrophils.

The aim of this study was to assess the simultaneous activation of MPO and 5-LOX in human neutrophils by taking the activity and product profile of 5-LOX under consideration. Therefore, different MPO-stimulants were investigated. Analysis was performed by reverse-phase high-performance liquid chromatography (RP-HPLC) and flow cytometry. Further attention was paid on cell vitality, MPO activation and redox status of stimulated neutrophils. We could demonstrate MPO-induced changes of 5-LOX product formation in neutrophils after pre-stimulation with H_2O_2 or phorbol 12-myristate 13-acetate (PMA) followed by addition of the calcium ionophore A23187.

Material and methods

Material

The chemicals were obtained from the following sources: aminophenyl fluorescein (APF) and HPLC-standards 5*S*-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HpETE), 5*S*-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HETE), 5*S*,12*R*-dihydroxy-6*E*,8*E*,10*E*,14*Z*-eicosatetraenoic acid (5,12-DiHETE), 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-oxo-ETE), LTB_4, prostaglandin B_2 (PGB_2) and AA were from Cayman Chemical (distributed by Biomol, Hamburg, Germany); HPLC solvents were from Carl Roth (Karlsruhe, Germany); glutathione colorimetric detection kit from Arbor Assays (Ann Arbor, Michigan, United States); biocoll (10 mM HEPES, 1.077 g/l, isotone) from Biochrom (Berlin, Germany) and all other chemicals including the JC-1 staining kit (CS0390) were from Sigma (Taufkirchen, Germany).

Working solutions of H_2O_2 were prepared by dilution of the corresponding stock solutions. Their concentrations were determined spectrophotometrically using $\varepsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$ [Beers and Sizer 1952]. The buffer system *hanks' balanced salt solution* (HBSS) with and without Ca^{2+} for the resuspension of neutrophils was prepared daily and adjusted to pH 7.4.

Isolation of neutrophil granulocytes

Neutrophils were isolated from heparinized peripheral human blood (10 U/ml), obtained from healthy volunteers after written consent, by dextran-enhanced sedimentation in the presence of 2% dextran for an hour, followed by a density centrifugation (biocoll 1.077 g/l, 20 °C, 400 g, 20 min). Remaining erythrocytes in the cell pellet were lysed by addition of one part distilled H_2O and incubation for 30 s. Then, five parts of HBSS without Ca^{2+} were added and after centrifugation this procedure was repeated. Neutrophils with a purity as well as vitality higher than 90% were isolated.

Determination of 5-LOX products from neutrophils by RP-HPLC

Neutrophils (5×10^6 cells/ml in HBSS with Ca^{2+}) were incubated in the presence or absence of the MPO inhibitor 4-aminobenzoic acid hydrazide (4-ABAH) with a final concentration of 2.5 mM for 15 min followed by stimulation with PMA or H_2O_2 for 20 min. During the last 5 min Ca^{2+} ionophore A23187 was added. All incubation steps took place at 37 °C in a final volume of 1 ml. Control experiments without the addition of inhibitor or stimulant were incubated for comparable duration. Afterwards, cell stimulation was stopped by addition of 1.01 ml ice-cold methanol containing 200 ng PGB_2 as internal standard. Samples were acidified with 30 µl of 1 N HCl, mixed for 30 s and incubated at 4 °C for 30 min. The precipitate was separated by centrifugation (4 °C, 10,000 g, 10 min) and supernatants were applied to C_{18} solid-phase extraction columns (100 mg, Discovery DSC-18 SPE Tube, Sigma, Taufkirchen, Germany) preconditioned with 1 ml methanol and 1 ml H_2O. The columns were washed with 1 ml H_2O and 1 ml H_2O/methanol (75/25, v/v). 5-LOX metabolites were eluted with 300 µl methanol. Extraction was performed by centrifugation (10 g, 4 °C). The samples were analyzed by RP-HPLC after maximal storage at -80 °C for 24 h. Here, a C_{18} column (Supelcosil LC-18-DB, 25 cm × 4.6 mm i.d., 5 µm) was used with an isocratic eluent consisting of acetonitrile/H_2O/acetic acid (60/40/0.2, v/v/v) and a flow rate of 1 ml/min. The eluate was monitored at 234 nm (quantification of conjugated dienes) and 270 nm (quantification of conjugated trienes). The HPLC device consisted of a Shimadzu liquid chromatographic system equipped with a Shimadzu LC-10ATvp isocratic solvent delivery system, Shimadzu SPD-10Avp dual wavelength absorbance detector, Shimadzu CTO-10ASvp column oven (35 °C) and Rheodyne injector with 20 µl loop volume. Major lipoxygenase

products (5-H(p)ETE, LTB_4 and 6-*trans*-LTB_4) were quantified using a calibration curve (13-point calibration) of the appropriate synthetic standards. Both forms of the non-enzymatic hydrolysis product 6-*trans*-LTB_4 (5*S*,12*S*/*R*-DiHETE) eluted at the same retention time and were measured as one product. LTB_4 was quantified using the 6-*trans*-LTB_4 calibration curve. Within each experiment, samples were referenced to the internal standard PGB_2.

Determination of cell vitality using JC-1

Cell vitality of neutrophils were assessed using the lipophilic, cationic dye JC-1 (J-aggregate forming cationic dye: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide). In vital cells JC-1 accumulates in mitochondrial matrix forming red fluorescent JC-1 aggregates. Upon changes of the mitochondrial electrochemical potential for example through the initiation of apoptosis, the JC-1 dye dispersed throughout the entire cell preventing JC-1 aggregate formation and leading to a shift to green fluorescence of JC-1 monomers. The cell vitality is defined as percentage of live, vital cells in the whole neutrophil population with intact membrane potential as assessed by red fluorescent JC-1 aggregates. For JC-1 staining 1×10^6 cells were resuspended in 500 µl HBSS without Ca^{2+} and mixed with 500 µl JC-1 staining buffer yielding a final concentration of 0.77 µM JC-1. As positive control valinomycin was added in a concentration of 4.3 µM that dissipates the mitochondrial electrochemical potential. Samples were stained with JC-1 at 37 °C for 10 min, washed with HBSS without Ca^{2+} (400 g, 5 min) and analyzed by flow cytometry.

Analysis of MPO activity by flow cytometry of APF-stained cells

Neutrophils (5×10^6 cells/ml in HBSS with Ca^{2+}) were incubated in the presence or absence of the MPO inhibitor 4-ABAH with a final concentration of 2.5 mM for 15 min. Afterwards, cells were stained with 10 µM APF for 15 min followed by 20 min stimulation with PMA or H_2O_2. During the last 5 min Ca^{2+} ionophore A23187 was added. All incubation steps took place at 37 °C. Thereafter, cells were washed by centrifugation (400 g, 5 min) and analyzed using flow cytometry.

Flow cytometry

Fluorescence intensity of cells was measured by FACSCalibur (Becton, Dickinson and Company, Franklin Lakes, New Jersey, United States) supplied with a 488 nm argon laser. Fluorescence of JC-1 monomers and aggregates was detected in FL1 channel (bandpass: 530 nm ± 15 nm) and FL2 channel (bandpass: 585 nm ± 21 nm), respectively. Both JC-1 monomer and aggregate fluorescence intensities were compensated. Fluorescence of APF stained cells was detected in FL1 channel (bandpass: 530 nm ± 15 nm). For each sample, 10^4 events were measured and results were analyzed using the software 'Flowing Software 2.5.1' by Perttu Terho (Cell Imaging Core, Turku Centre for Biotechnology, Finland).

Quantification of total glutathione

Neutrophils (5 × 106 cells/ml in HBSS with Ca2+) were incubated as described earlier (Determination of 5-LOX products from neutrophils by RP-HPLC) for simultaneous activation of 5-LOX and MPO. Then, cells were washed with HBSS without Ca2+ for two times (400 g, 5 min) and resuspended in 50 µl HBSS without Ca2+. The measurement of glutathione (GSH) based on the quantification of free thiol groups after protein precipitation. Thereby, total glutathione was measured using a glutathione reductase/NADPH-system. Oxidized glutathione (GSSG) was quantified after blockage of free glutathione using 1-methyl-2-vinylpyridinium triflate (MVP) added directly after sample

generation. Therefore, each sample was divided to two parts. MVP was applied to one part in a final concentration of 1 mM. Equal amount of H2O was added to the other part. Afterwards, samples were immediately stored at -80 °C. For glutathione quantification samples were mixed with 200 µl 5% 5-sulfo-salicylic acid dehydrate solution and precipitate and supernatant were separated by centrifugation (4 °C, 13,000 g, 10 min). Supernatant was diluted four-fold with assay buffer and transferred to a 96-well plate. Hereinafter, colorimetric detection assay mixture and the glutathione reductase/NADPH-system, obtained from Arbor Assays (K0006-H1, Ann Arbor, Michigan, United States), were added to the sample and absorbance at 405 nm was measured for 15 min using the microplate reader Tecan Infinite 200 PRO (Männedorf, Switzerland). Oxidized glutathione (GSSG) was detected in samples with MVP blocking any free GSH in the sample. The samples that have not been treated with MVP were used to measure total glutathione. The amount of free GSH in the sample was calculated from the difference between total glutathione and GSH generated from GSSG. The standard curve was prepared with GSSG also in two parts, whereby to one part MVP was transferred comparable to the sample mixture.

Statistical analysis

The data are reported as means ± standard deviation of at least three independent experiments. Statistical evaluation of the data was performed by Student's two-tailed t test for independent means to compare treated samples and the appropriate control.

Results

Stimulation of neutrophils for 5-LOX activation

Human neutrophils were stimulated with increasing concentrations of the calcium ionophore A23187 (Ca^{2+}-mobilizing agent). Here, the 5-LOX activity for endogenous arachidonic acid was assessed by RP-HPLC (Figure 1 A).

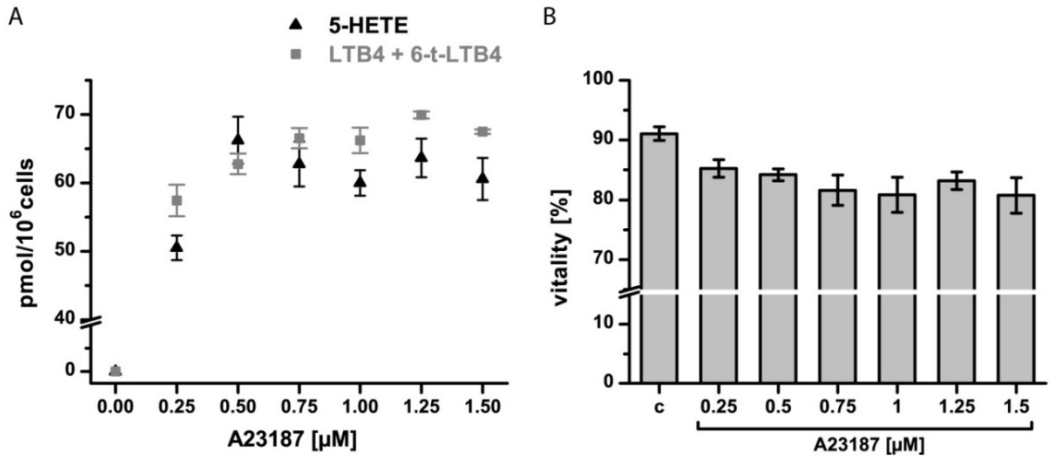

Figure 1: Stimulation of neutrophils with increasing concentrations of A23187. Neutrophils (5×10^6 cells/ml in HBSS with Ca2+, pH 7.4) were incubated at 37 °C for 30 min and were subsequently stimulated with A23187 for 5 min. (A) 5-LOX product formation was determined by RP-HPLC (n = 3). (B) Vitality of unstimulated (control - c) and A23187-treated cells was assessed by JC-1 staining (n = 3).

In contrast to cell-free experiments with recombinant 5-LOX, where 5-HpETE and 6-*trans*-LTB_4 are the main products, neutrophils produced 5-HETE, the glutathione peroxidase-catalyzed reduction product of 5-HpETE, and a mixture of LTB_4 + 6-*trans*-LTB_4. In cellular experiments no 5-HpETE could be measured assuming that all 5-HpETE is reduced to 5-HETE during neutrophil activation. The LTB_4 is produced from LTA_4 by LTA_4 hydrolase, whereas 6-*trans*-LTB_4 (5*S*,12*S*/*R*-DiHETE) originates from non-enzymatic LTA_4-hydrolysis. With increasing A23187 concentrations a saturation of the 5-LOX product formation of 5-HETE and LTB_4 + 6-*trans*-LTB_4 was measured yielding around 130 pmol 5-LOX products per 1×10^6 cells, whereby similar amounts of 5-HETE and leukotrienes products were formed. The pre-incubation with the 5-LOX inhibitor BWA4C resulted in a complete diminution of 5-LOX product formation (data not shown). Furthermore, vitality of the neutrophils was determined using JC-1 staining (Figure 1 B). Due to A23187 stimulation a slight decrease in cell vitality to 80% compared to the unstimulated control with 90% vitality was observed. To ensure an effective 5-LOX activation, neutrophils were stimulated with 0.75 µM A23187 in the subsequent experiments.

For optimal 5-LOX stimulation it became apparent that the incubation time of neutrophils with A23187 is a critical parameter. Both 5-LOX product composition (Figure 2 A) and cell vitality (Figure 2 B) showed drastic changes in dependence on the duration of A23187 stimulation. Especially, the amount of 5-HETE greatly decreased at longer stimulation. Furthermore, the amount of LTB_4 was reduced after 20 min compared to the maximal concentration at 5 min. The 6-*trans*-LTB_4 concentration showed a constant increase at longer incubation times. Interestingly, the longer A23187 stimulation resulted in a considerable decline of cell vitality to 65% after 20 min. To ensure a maximal formation of

5-HETE and LTB_4, cells were stimulated for 5 min with 0.75 µM A23187 in the subsequent experiments.

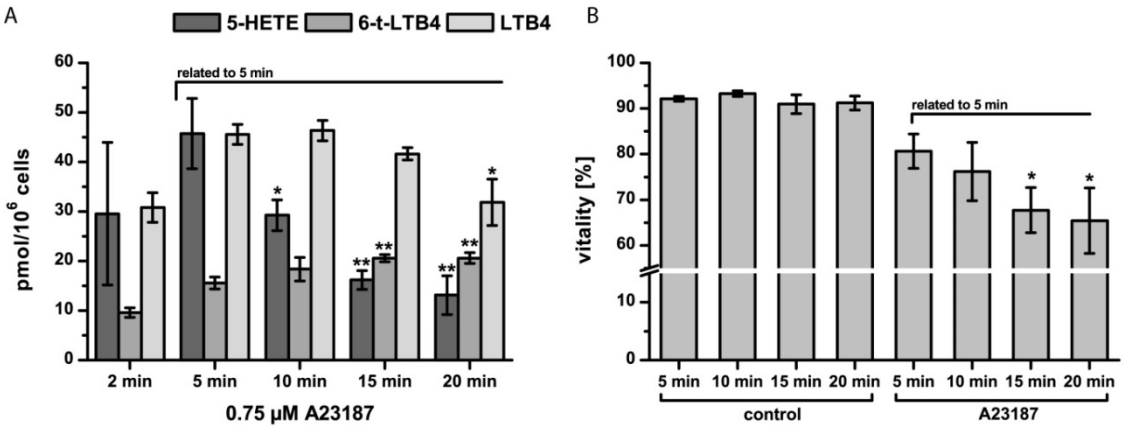

Figure 2: Stimulation of neutrophils with A23187 as a function of incubation time. Neutrophils (5×10^6 cells/ml in HBSS with Ca^{2+}, pH 7.4) were incubated at 37 °C for 30 min and were subsequently stimulated with 0.75 µM A23187 for 5 - 20 min. (A) 5-LOX product formation was determined by RP-HPLC (n = 3). (B) Vitality of unstimulated (control) and A23187-treated cells, differing in their incubation time, was assessed by JC-1 staining (n = 3). A two-tailed *t* test (* $p \leq 0.05$; ** $p \leq 0.01$) was performed against the appropriate 5 min sample.

Simultaneous stimulation of 5-LOX and MPO in neutrophils using H_2O_2 and A23187

Neutrophils were stimulated by a direct supply of H_2O_2 to evaluate the effect of MPO on the 5-LOX activity. The permeability of extracellular added H_2O_2 across the neutrophil membrane is relatively high [Winterbourn *et al.* 2006], so it can be assumed that enough H_2O_2 can get inside the cell. A part of this intracellular amount of H_2O_2 could be decomposed by neutrophil catalase or further peroxidases [Roos *et al.* 1980], before reaching the MPO. For this reason the activity of MPO was measured under these conditions (Figure 3 A) with the non-fluorescent dye APF yielding fluorescein after reaction with HOCl [Flemmig *et al.* 2012; Flemmig *et al.* 2015]. Here, the APF-derived fluorescence signal of H_2O_2-stimulated cells increased in comparison to A23187-stimulated cells. Thereby, 2 mM H_2O_2 enhanced the APF-derived fluorescence by 8.1-times compared to A23187-stimulated cells. Further controls with the MPO inhibitor 4-ABAH were considered for calculation of the ratio of APF fluorescence intensity of stimulated cells without 4-ABAH compared to 4-ABAH treated cells. This was necessary because also hydroxyl radicals or peroxynitrite could oxidize APF [Setsukinai *et al.* 2003]. Interestingly, the ratio of APF fluorescence in the absence and presence of 4-ABAH revealed in A23187-stimulated cells without the addition of H_2O_2 a 6.2-fold MPO-induced APF increase (Figure 3 B). Indeed, addition of H_2O_2 resulted in considerable rise of the MPO-induced APF response. For example, cells treated with 1.5 mM H_2O_2 showed a 3.3-times higher MPO-induced APF response compared to the A23187 control (Figure 3 B). Also the vitality of H_2O_2-stimulated cells was examined (Figure 3 C). Even the highest applied H_2O_2 concentration did not significantly reduce the neutrophil vitality.

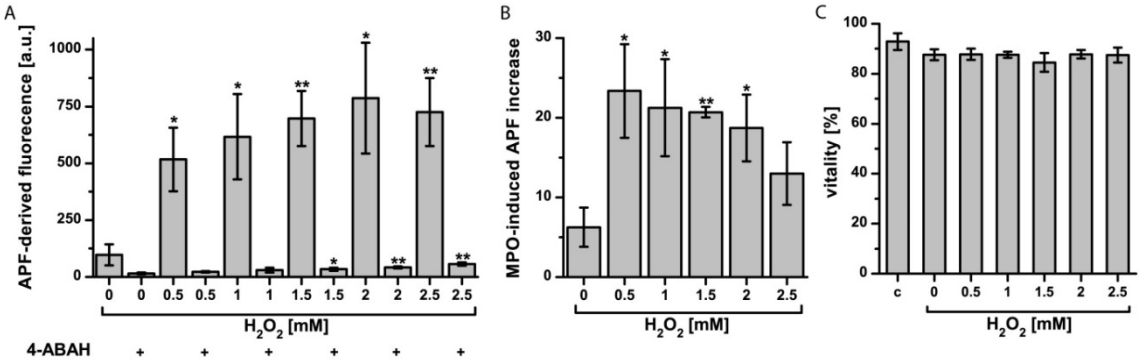

Figure 3: Determination of MPO activity and cell vitality in A23187- and H_2O_2-stimulated neutrophils. Neutrophils (5×10^6 cells/ml in HBSS with Ca^{2+}, pH 7.4, 37 °C) were incubated with and without 2.5 mM 4-ABAH for 15 min. Afterwards, cells were stained with 10 µM APF for 15 min and stimulated with H_2O_2 for 20 min. During the last 5 min A23187 (0.75 µM) was added in all samples. For cell vitality (C) APF staining was omitted. Samples were washed with HBSS without Ca^{2+} and analyzed by flow cytometry. (A) APF-derived fluorescence intensity of A23187-treated sample and H_2O_2+A23187-stimulated cells were measured (n = 3). (B) The ratio of APF fluorescence intensity of stimulated cells without 4-ABAH to 4-ABAH treated cells was calculated. (C) Cell vitality of unstimulated (control - c) and A23187- and H_2O_2+A23187-stimulated cells was assessed by JC-1 staining (n = 3). A two-tailed *t* test (* $p \leq 0.05$; ** $p \leq 0.01$) was performed against A23187 or A23187+4-ABAH.

In the next step, the activity of 5-LOX was measured under these conditions (Figure 4). Interestingly, increasing concentrations of H_2O_2 led to a slightly increase in 5-HETE. For example applying 2 mM H_2O_2 resulted in 1.22-fold higher 5-HETE concentration (Figure 4 A). In contrast, the same amount of H_2O_2 reduced the formation of LTB_4 and 6-*trans*-LTB_4 to 44% and 37%, respectively (Figure 4 B). The inhibition of MPO by 4-ABAH clearly showed that the H_2O_2-induced increase in 5-HETE depends on the MPO-activity. Because, in the presence of 4-ABAH there was 50% lower amounts of 5-HETE in neutrophils stimulated with 2 mM H_2O_2. For unstimulated neutrophils a part of the amount of 5-HETE (30%) also depended on the MPO activity. This is in line with the detected MPO activity in A23187-stimulated cells assessed by APF staining (Figure 3 B).

These results reflect properly the *in vitro* experiments, where MPO-derived oxidants showed a considerable impact on recombinant human 5-LOX, impairing the epoxidation of 5-HpETE, whereas the hydroperoxidation of arachidonic acid was unaffected [Zschaler *et al.* 2015]. Unfortunately, a direct effect of MPO on the H_2O_2-mediated reduction of LTB_4 could not be evaluated using 4-ABAH (Figure 4 B). Only for the neutrophil stimulation with 0.5 mM H_2O_2 a 16% higher LTB_4 amount was measured indicating a weak MPO-induced LTB_4-reduction, which was abrogated after 4-ABAH treatment. However, in the other samples no influence of 4-ABAH treatment occurred. This could be interpreted by a H_2O_2-driven inhibition of 5-LOX activity. In previous experiments the incubation of recombinant 5-LOX with H_2O_2 also diminished its activity [Zschaler *et al.* 2015]. However, this does not explain why the 5-HETE concentration was unaffected. Therefore the reduction of LTB_4 could be a superimposition of a H_2O_2 effect inhibiting 5-LOX and a MPO-driven HOCl effect on 5-LOX yielding to a reduction of the amount of LTB_4 and 6-*trans*-LTB_4, while the amount of 5-HETE slightly increased. Thus, in the presence of the MPO inhibitor 4-ABAH the reduction of LTB_4 is caused by H_2O_2. This could be compared with further *in vitro*

experiments, where both human MPO and 5-LOX, were co-incubated, and a H_2O_2-generating system activated the peroxidase [Zschaler *et al.* 2015].

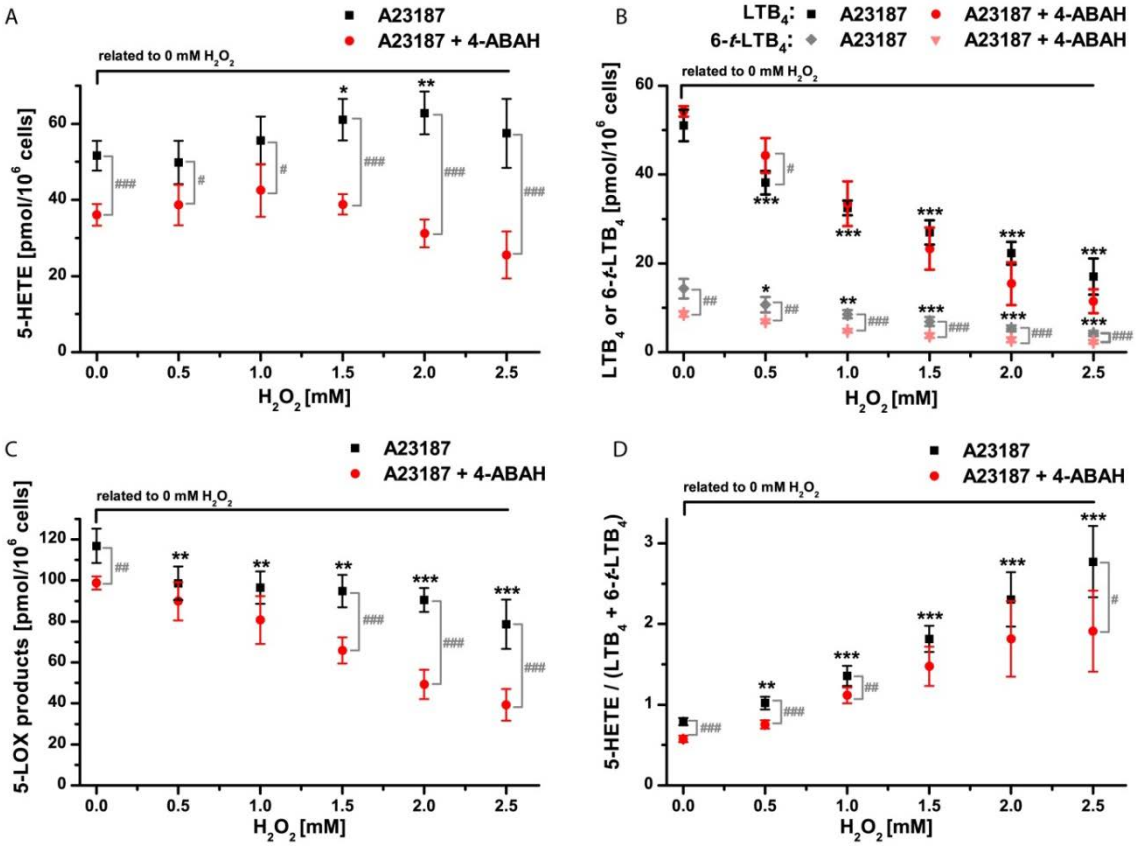

Figure 4: Stimulation of neutrophils with A23187 and H_2O_2 for simultaneous activation of 5-LOX and MPO. Neutrophils (5×10^6 cells/ml in HBSS with Ca^{2+}, pH 7.4, 37 °C) were incubated with and without 2.5 mM 4-ABAH for 15 min. Afterwards cells were stimulated with H_2O_2 for 20 min and during the last 5 min A23187 (0.75 µM) was added. The amount of 5-HETE (A), LTB_4 or 6-*trans*-LTB_4 (B) and total 5-LOX product formation (C) was determined by RP-HPLC (n = 4-6). Furthermore, the ratio of 5-HETE to the sum of LTB_4 + 6-*trans*-LTB_4 (D) was related to the applied H_2O_2 concentration. A two-tailed *t* test was performed. Grey hashtags indicate the correlation of samples with and without inhibitor 4-ABAH (# $p \leq 0.05$; ## $p \leq 0.01$; ### $p \leq 0.001$). Black asterisks indicate the correlation of the H_2O_2-treated samples without inhibitor to the untreated sample without H_2O_2 (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

In considering an effect of 4-ABAH on the amount of 6-*trans*-LTB_4 a decrease after MPO inhibition was present, albeit in a relatively low quantity compared to the overall 5-LOX product amount. This could be explained in part with higher availability of H_2O_2 in case of MPO inhibition, yielding a stronger effect after 4-ABAH treatment.

In addition, the whole 5-LOX activity was summarized after MPO stimulation (Figure 4 C). The stimulation with H_2O_2 resulted in a maximal decrease of 33% of 5-LOX product formation, whereas in 4-ABAH treated samples, H_2O_2 induced a maximal decline of 60%. As shown above this decrease is due to the drastic reduction of LTB_4 and 6-*trans*-LTB_4. This is also reflected in a strong rise of the ratio of 5-HETE to the sum of LTB_4 + 6-*trans*-LTB_4 also increasing after MPO inhibition, however, to a lower extent (Figure 4 D). Control experiments with solely H_2O_2-treated neutrophils without the addition of A23187 revealed no 5-LOX activity (data not shown).

The stimulation of neutrophils with H_2O_2, under consideration of cell vitality and MPO activity, changed the product profile of 5-LOX, leading to the dominance of 5-HETE in relation of LTB_4 and 6-*trans*-LTB_4. This became apparent in the increased formation of 5-HETE after MPO activation in dependence on 4-ABAH addition. For LTB_4 and 6-*trans*-LTB_4 a MPO-induced reduction could not be clearly shown, possibly due to a superimposition with a H_2O_2-mediated inhibition of 5-LOX activity. Especially in the presence of 4-ABAH, which disturbs the clearance of external supplied H_2O_2 by neutrophil MPO.

Simultaneous stimulation of 5-LOX and MPO in neutrophils using PMA and A23187

Besides the direct supply of H_2O_2, the MPO can also be activated by initiation of the respiratory burst. As a further neutrophil stimulant PMA was used. PMA is a known activator of protein kinase C and is able to increase reactive oxygen species (ROS) production in neutrophils by NADPH oxidase stimulation [Nauseef *et al.* 1991]. First of all the MPO activity was measured under these conditions with APF (Figure 5).

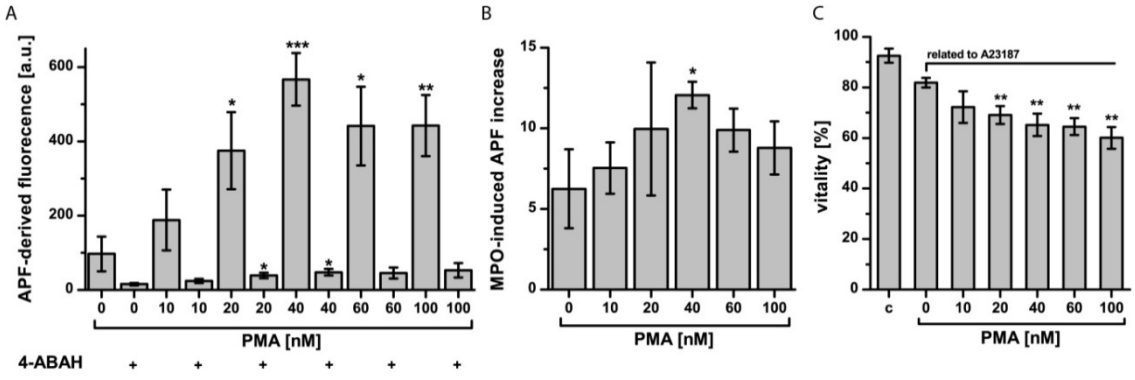

Figure 5: Determination of MPO activity and cell vitality in A23187- and PMA-stimulated neutrophils. Neutrophils (5×10^6 cells/ml in HBSS with Ca^{2+}, pH 7.4, 37 °C) were incubated with and without 2.5 mM 4-ABAH for 15 min. Afterwards, cells were stained with 10 µM APF for 15 min and stimulated with PMA for 20 min. During the last 5 min A23187 (0.75 µM) was added in all samples. For cell vitality (C) APF staining was omitted. Samples were washed with HBSS without Ca^{2+} and analyzed by flow cytometry. (A) APF-derived fluorescence intensity of A23187-treated sample and PMA+A23187-stimulated cells were measured (n = 3). (B) The ratio of APF fluorescence intensity of stimulated cells without 4-ABAH to 4-ABAH treated cells was calculated. (C) Cell vitality of unstimulated (control - c) and A23187- and PMA+A23187-stimulated cells was assessed by JC-1 staining (n = 3). A two-tailed *t* test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) was performed against A23187 or A23187+4-ABAH.

Here, PMA (40 nM) strongly enhanced the APF-derived fluorescence signal by 5.7-times compared to the sole A23187-treated control (Figure 5 A). However, further controls with the MPO inhibitor 4-ABAH were considered for calculation of the ratio of APF fluorescence intensity of stimulated cells without 4-ABAH to 4-ABAH treated cells. This MPO-induced APF response revealed only a two-fold increase after stimulation with 40 nM PMA compared to the A23187-treated control (Figure 5 B). Higher PMA concentrations did not result in a stronger MPO-activation. Under these harsh conditions, we cannot exclude the formation of hydroxyl radicals, which are also capable to react with APF [Setsukinai *et al.* 2003]. Higher amounts of PMA were not applied, because already 100 nM PMA led to a strong decline of cell vitality (Figure 5 C). In this case, only 60% vital cells were determined.

Subsequently, the activity of 5-LOX was measured under these conditions. Control experiments were performed with cells stimulated with PMA alone or the used vehicle DMSO. In both cases there was no 5-LOX activity (data not shown).

The treatment of neutrophils with increasing concentrations of PMA resulted in a drastic decline of the amount of 5-HETE (Figure 6 A).

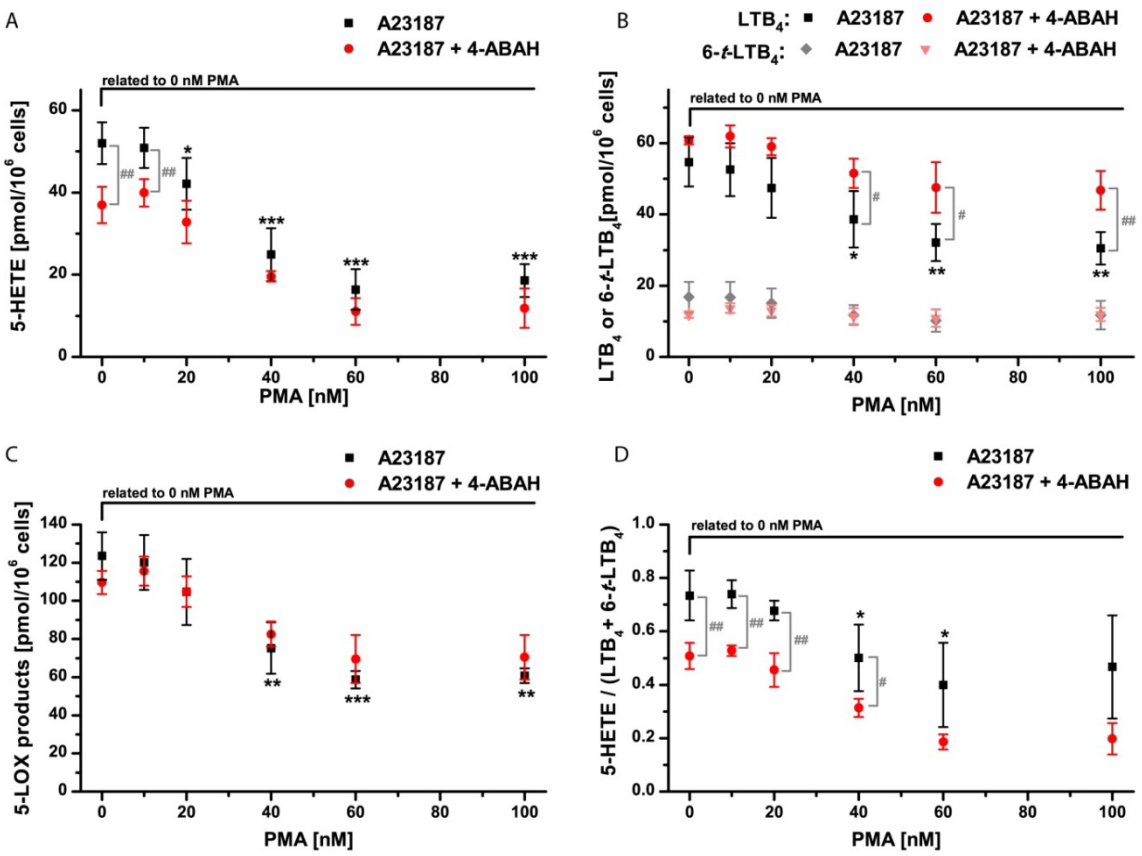

Figure 6: Stimulation of neutrophils with A23187 and PMA for simultaneous activation of 5-LOX and MPO. Neutrophils (5×10^6 cells/ml in HBSS with Ca^{2+}, pH 7.4, 37 °C) were incubated with and without 2.5 mM 4-ABAH for 15 min. Afterwards cells were stimulated with PMA for 20 min and during the last 5 min A23187 (0.75 µM) was added. The amount of 5-HETE (A), LTB_4 or 6-*trans*-LTB_4 (B) and total 5-LOX product formation (C) was determined by RP-HPLC (n = 3-4). Furthermore, the ratio of 5-HETE to the sum of LTB_4 + 6-*trans*-LTB_4 (D) was related to the applied PMA concentration. A two-tailed *t* test was performed. Grey hashtags indicate the correlation of samples with and without inhibitor 4-ABAH (# $p \leq 0.05$; ## $p \leq 0.01$). Black asterisks indicate the correlation of the PMA-treated samples without inhibitor to the untreated sample without PMA (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

Here, for example the stimulation with 60 nM PMA led to 68% lower concentrations of 5-HETE. The amount of LTB_4 (Figure 6 B) was only reduced by 41% after application of 60 nM PMA. The inhibition of MPO by 4-ABAH has two different consequences. The 5-HETE producing activity was reduced by 4-ABAH in the A23187-treated sample and in cells stimulated with 10 nM PMA. However, the drastic PMA-induced decrease in 5-HETE was not affected by MPO inhibition. In contrast, higher amounts of LTB_4 were detected when MPO was inhibited and neutrophils were stimulated with PMA. Whereby, the LTB_4 concentration increased by 50%, when cells were treated with 4-ABAH and subsequently stimulated with 60-100 nM PMA. For 6-*trans*-LTB_4 no PMA or 4-ABAH effect was observed (Figure 6 B).

Comparing the stimulation of neutrophils with PMA and H_2O_2 regarding the 5-LOX activity revealed drastic differences. PMA resulted in an abrogation of the partially MPO-mediated production of 5-HETE in unstimulated cells due to the strong decrease in the amount of 5-HETE clearly distinguishing it from the stimulant H_2O_2 used for MPO activation. On that basis it is striking that the LTB_4 reduction after PMA stimulation depended on the MPO activity. Here, higher amounts of LTB_4 were seen in 4-ABAH-treated neutrophils. It could be assumed, that active MPO reduced the production of LTB_4 after PMA stimulation. As mentioned above the stimulation with PMA resulted in a gradual impairment of cell vitality (Figure 5 C). A partial impairment of 5-LOX activity due to reduced cell functionality could be assumed.

Reflecting the total 5-LOX product formation a strong decrease of 50% was observed after application of 100 nM PMA (Figure 6 C). The addition of 4-ABAH had no influence on the overall extent of 5-LOX products. However, the ratio of 5-HETE to the sum of LTB_4 + 6-*trans*-LTB_4 was changed in consequence of 4-ABAH (Figure 6 D). Both the A23187-treated sample and cells stimulated with low PMA concentrations showed a reduced ratio of 5-HETE to the sum of LTB_4 + 6-*trans*-LTB_4 when MPO activity was suppressed with 4-ABAH due to the possibly MPO-mediated formation of 5-HETE in unstimulated cells. In addition, 40 nM and 60 nM PMA led to a reduction of the ratio of 5-HETE to the sum of LTB_4 + 6-*trans*-LTB_4 reflecting the dominance of LTB_4 in PMA-stimulated neutrophils. The addition of 4-ABAH to cells stimulated with PMA in the range between 40 and 100 nM resulted in a decrease in the ratio of 5-HETE to the sum of LTB_4 + 6-*trans*-LTB_4, albeit with low significance.

Quantification of total glutathione

In the presence of active 5-LOX the stimulation of neutrophils with H_2O_2 or PMA revealed different changes of 5-LOX product spectrum. Both stimulants were primarily used for MPO-activation. To explore the accompanying alteration of the neutrophil redox state, the amount of oxidized and reduced GSH was determined (Figure 7). Here, in unstimulated sample 900 pmol per 1×10^6 cells total glutathione were measured composed of oxidized and reduced GSH comparable to previous reports [Graham *et al.* 2009]. However, there was no GSSG detected, possibly too low concentrations were present. According to the standard curve the lowest measurable GSSG concentration was 80 pmol per 1×10^6 cells.

The stimulation with A23187 led to a reduction of total glutathione to 690 pmol. The further addition of PMA resulted in a decrease of 40% compared to the A23187 stimulated cells. However, there was no dependence on PMA concentration. The treatment with H_2O_2 revealed a lower impact on total glutathione. In case of 0.5 mM H_2O_2 no significant changes were apparent. Only 1.5 mM or 2.5 mM H_2O_2 resulted in reduction of 20% of total glutathione. Despite the loss of GSH both stimulants PMA and H_2O_2 did not increase the amount of GSSG assuming the presence of further GSH oxidation products.

It can be summarized that neutrophil stimulation with PMA lead to more drastic changes of redox status than H_2O_2. A reduction of GSH could have the consequence of a reduced activity of glutathione peroxidase (GpX) and an impaired reduction of 5-HpETE to 5-HETE. This in turn elucidates why the amount of LTB_4 was reduced to a lower extent. However the PMA induced changes of 5-LOX activity cannot be solely explained with variation in total glutathione. A reduced activity of glutathione peroxidase was also associated with a higher hydroperoxide tone, which is essential for 5-LOX activation [Hatzelmann *et al.* 1989].

Nevertheless, the different behavior of PMA and H_2O_2 regarding 5-LOX product profile could be partially related to the neutrophil redox status.

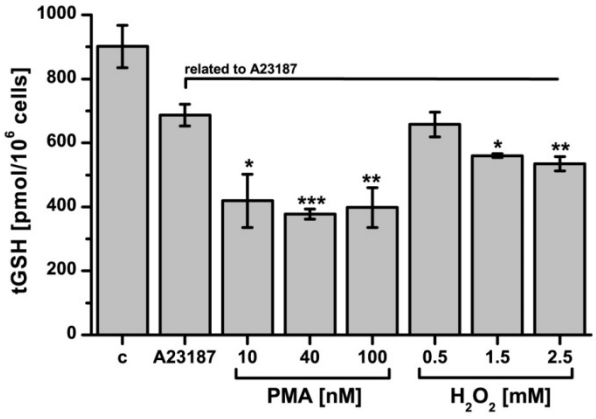

Figure 7: Determination of total glutathione (tGSH) amount in stimulated cells. Neutrophils (5×10^6 cells/ml in HBSS with Ca^{2+}, pH 7.4) were incubated at 37 °C for 15 min. Afterwards cells were stimulated with PMA or H_2O_2 for 20 min. During the last 5 min A23187 (0.75 µM) was added. All incubation steps were performed at 37 °C. Samples were washed with HBSS without Ca^{2+} and total glutathione of unstimulated (control - c) and A23187- and H_2O_2- or PMA-stimulated cells were measured (n = 3). A two-tailed *t* test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) was performed against A23187.

Discussion and conclusion

The simultaneous activation of MPO and 5-LOX in neutrophils revealed an effect of MPO on the 5-LOX product formation differing in the applied cell stimulant. In this study, special attention was drawn to cell vitality assessed by the dye JC-1 serving as an early marker for changes in the mitochondrial potential. An incubation of neutrophils with the Ca^{2+} ionophore A23187 (0.75 µM) for more than 10 min resulted in a considerable decrease in cell vitality and reduced formation of specific 5-LOX products. Thus, we choose an incubation time of 5 min with A23187 (0.75 µM) for most experiments. Under these conditions at least 80% vital cells were detected. Our experiments revealed a comparable amount of 5-LOX products as previously reported applying 2.5 µM A23187 for 5 min [Werz *et al.* 2002]. The Ca^{2+} ionophore A23187 increased the cytoplasmic Ca^{2+} level, and favors, thus, the translocation of 5-LOX to the nuclear envelope [Werz *et al.* 2002]. Furthermore, elevated Ca^{2+} concentrations regulate the activity of $cPLA_2$ activity [Gijon and Leslie 1999], a critical step for stimulus dependent AA mobilization. The 5-LOX activity on endogenous AA was sufficient for assessment of 5-LOX product formation in our case. In contrast, the combined addition of exogenous AA (20 - 40 µM) and A23187 was accompanied by a reduced cell vitality and a higher yield of 5-HETE compared to LTB_4 fractions (unpublished data).

Application of external H_2O_2 for MPO activation has several consequences on 5-LOX product formation by neutrophils. First of all, there was a considerable increase in the ratio of 5-HETE to the sum of LTB_4 + 6-*trans*-LTB_4 with increasing H_2O_2 concentrations. On the one hand, this was caused mainly by the drop of both LTB_4 fractions. On the other hand, 5-HETE increased slightly under these conditions. The inhibition of MPO reduced most of all the level of 5-HETE, whereby the effect increased with higher amounts of H_2O_2. It could be assumed that MPO metabolites mainly impair the epoxidation reaction of 5-HpETE in comparison to the hydroperoxidation reaction of AA. Similar observations were previously obtained for the effect of HOCl and HOBr on recombinant 5-LOX [Zschaler *et al.* 2015].

However, it should be stressed that inhibition of MPO did not counteract the overall decrease in LTB_4 + 6-*trans*-LTB_4. Although, for LTB_4 there was a slight increase at low H_2O_2, in the presence of higher H_2O_2 no significant changes were detected. For 6-*trans*-LTB_4 there was a diminution in concentration. According to our previous results of recombinant human 5-LOX incubated with the MPO-H_2O_2-Cl^- system [Zschaler *et al.* 2015] a superimposition of a MPO-driven HOCl effect on 5-LOX, resulting in a reduction of the product formation of LTB_4 and 6-*trans*-LTB_4, and a H_2O_2-mediated inhibition of 5-LOX activity, could be assumed. In neutrophils, H_2O_2 is mainly consumed by catalase, myeloperoxidase, and GpX. The inhibition of MPO by 4-ABAH enhanced considerably the H_2O_2-mediated decrease in 5-LOX products. This was obvious in the further decrease in 6-*trans*-LTB_4 after 4-ABAH treatment. In the previous study [Zschaler *et al.* 2015] the recombinant 5-LOX was incubated with a glucose oxidase-glucose (GOD-Glu)-system producing H_2O_2 for MPO activation revealing a HOCl-mediated effect on 5-LOX product formation. However, in the absence of the MPO substrate Cl^-, H_2O_2 generated by GOD-Glu system inhibited 5-LOX [Zschaler *et al.* 2015]. These *in vitro* experiments revealed comparable results to the observed product changes of 5-LOX in neutrophils after simultaneous activation of MPO. An impact of MPO products on LTA_4 hydrolase activity is also possible, influencing the ratio of 6-*trans*-LTB_4 to LTB_4. However, more detailed investigations are necessary to draw a final conclusion. It can be concluded that the activation of MPO by H_2O_2 increased the 5-HETE formation and changed

the product ratio of 5-LOX with lower LTB_4 + 6-*trans*-LTB_4 formation comparable to the incubation of MPO and 5-LOX in *in vitro* experiments.

In order to further clarify this point, PMA was used as MPO stimulant. Here, varying 5-LOX product changes appeared as described for H_2O_2. The most marked difference was the strong reduction of 5-HETE formation observed at PMA concentrations higher than 20 nM. Effects caused by MPO were less expressed in comparison to H_2O_2. There was a slightly lower formation of 5-HETE in unstimulated cells after MPO inhibition. However, increasing PMA concentrations diminished this MPO effect. The amount of LTB_4 was also reduced in PMA-treated neutrophils, albeit to a lower extent compared to 5-HETE implying the reduced ratio of 5-HETE to the sum of LTB_4 + 6-*trans*-LTB_4. Interestingly, the formation of LTB_4 depended on the activity of MPO after stimulation with higher PMA concentrations. It could be assumed that, in this case, a HOCl-mediated effect is only obvious for LTB_4, whereas a further MPO-independent process diminished the amount of 5-HETE. Oxidative stress could induce the oxidation of 5-HETE to 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-oxo-ETE) by a dehydrogenase [Graham *et al.* 2009; Powell and Rokach 2013]. However, we detected no increase in 5-oxo-ETE formation, hence an oxidation of 5-HETE can be excluded. The quantification of the LTB_4 ω-oxidation products, 20-OH-LTB_4 and 20-COOH-LTB_4 emerging as further minor products, was not possible. Indeed, the qualitative assessment of this double peak revealed no influence after MPO inhibition with 4-ABAH. The different effect of MPO inhibition on 6-*trans*-LTB_4 and LTB_4, both generated from LTA_4, cannot be explained. Again, a modulation of the LTA_4 hydrolase activity cannot be excluded.

The differences of PMA and H_2O_2 stimulation in relation to 5-LOX product formation were further assessed in the light of the redox status by means of total glutathione quantification. The amount of total glutathione decreased after stimulation with A23187. The additional presence of H_2O_2 resulted only in minor changes, whereas PMA reduced total glutathione to greater extent. An associated increase in oxidized GSSG was not detected. Graham *et al.* determined a comparable amount of GSH in unstimulated human neutrophils, while in aging neutrophils half of GSH was measured and 3% GSSG was present [Graham *et al.* 2009]. This reduction of GSH was comparable to the stimulation with PMA in our experiments. Therefore, it can be assumed that these low changes of GSSG are below the detection limit of the applied method. The loss of GSH can be attributed to the formation of further oxidation products in addition to GSSG that will be not detected by the assay system. Furthermore, PMA was shown to induce GSH-S-thiolation of human neutrophil proteins during respiratory burst contributing to reduced GSH level [Chai *et al.* 1994].

The reduced amount of GSH could result in a lower activity of GpX and a reduced formation of 5-HETE from 5-HpETE. Accordingly, the higher level of 5-HpETE can serve as an activator of 5-LOX, and can favor the epoxidation reaction. We really observed a preferential formation of LTB_4 compared to 5-HETE with increasing PMA. Nevertheless, the total amount of LTB_4 also decreased in a concentration-dependent manner. This could be explained with a higher oxidative stress in neutrophils and reduced cell vitality due to PMA impairing 5-LOX activity towards endogenous AA. Another aspect concerning redox properties of GSH serves attention. The reaction potential of the redox couple GSSG/2 GSH depends not only on the activity ratio between the reduced and oxidized forms as in the case of most other redox couples but also on the concentration of GSH [Schafer and

Buettner 2001]. Thus, a more reduced GSH level in PMA-stimulated cells might much stronger affect the activity of GpX as in H_2O_2-stimulated cells.

In different previous studies stimulation of neutrophils with PMA in addition to A23187 was related to 5-LOX activity. Direct comparisons between these results are difficult due to variable experimental conditions. Werz *et al.* showed that in neutrophils, pre-stimulated with PMA, the leukotriene biosynthesis was up-regulated [Werz *et al.* 2001]. This was explained by a higher release of AA and an increased phosphorylation and translocation of 5-LOX. However, the 5-LOX product formation was not evaluated individually, so no conclusion about the product ratio can be drawn. These observations based mainly on the application of suboptimal concentrations of A23187 (0.1 µM). Also an increased release of LTB_4 by neutrophils synergistically stimulated with PMA and A23187 (0.4 µM) was depicted by Liles *et al.* [Liles *et al.* 1987]. During these studies only the amount of LTB_4 and its metabolites were quantified. Interestingly, the amount of LTB_4 induced by higher concentration of A23187 alone (1 µM) was not significantly different from that induced by A23187 (0.4 µM) plus PMA.

Contrary to this, the pre-incubation with 16-160 nM PMA in addition to A23187 (2 µM) suppressed the recovery of all 5-LOX products by neutrophils from healthy individuals, whereas neutrophils from patients with chronic granulomatous disease (CGD) were unaffected [Hamasaki *et al.* 1989]. This decrease was attributed to increased ROS production by PMA that may directly affect endogenous LTB_4 and convert it to metabolites distinct from ω-oxidations products. However, no such oxidations products derived from LTB_4 or 5-HETE were described. We also applied an optimal A23187 concentration and measured a reduction in LTB_4 after incubation with PMA. The treatment with the MPO-inhibitor 4-ABAH allows the hypothesis of a partially MPO-induced LTB_4 reduction.

In conclusion, the activation of MPO by H_2O_2 or PMA is able to affect the product profile of 5-LOX in human neutrophils. The pre-stimulation with H_2O_2 resulted in a concentration-dependent increase in the ratio of 5-HETE to the sum of LTB_4 + 6-*trans*-LTB_4. Whereby, the diminution of LTB_4 in PMA-stimulated cells was partially dependent on MPO activity. This coincides well with effects of HOCl, HOBr and the MPO-H_2O_2-Cl^- system on the product profile of recombinant 5-LOX [Zschaler *et al.* 2015]. Any reaction of HOCl and AA metabolites could be excluded. Indeed, as previously shown the incubation of HOCl with an isomer of 5-HETE resulted in the formation of chlorohydrin products, which could be also assumed for LTB_4. However, in the presence of potential reaction partners of HOCl, as the thioether group of methionine or other amino acids the formation of chlorohydrin products was disturbed [Zschaler and Arnhold 2014]. In line with this, Lee *et al.* demonstrated in a reaction of LTC_4 with HOCl, that the thioether group is the primary target of HOCl. Thereby, a S-chlorosulfonium ion decomposed into two diastereoisomers of LTC_4 sulfoxide and of 6-trans-LTB_4, but only in the absence of further amino acids scavengers [Lee *et al.* 1982]. Therefore the 5-LOX enzyme represents a better target site for HOCl than their products. Thus, HOCl production could have further microbicidal-independent effects in neutrophils. Another example is the formation of HOCl-derived chloramines that can modulate the activation of NF-κB by oxidation of IκBα [Gloire *et al.* 2006]. Interestingly, the incubation of recombinant 5-LOX with monochloramine revealed an increase in the 5-H(p)ETE to 6-*trans*-LTB_4 ratio comparable to HOCl (unpublished results). Therefore, also further HOCl-derived oxidants in neutrophils, like the hydrophobic monochloramine, could modulate 5-LOX activity. In this study the main focus was directed on adequate MPO activators. To further

elucidate the simultaneous activation of MPO and 5-LOX more physiological stimulants should be tested for influence on 5-LOX product profile.

Authorship

JZ and JA initiated the study's concept and wrote the manuscript. JZ performed the experiments and analyzed the data.

Conflict of interest disclosure

The authors declare no conflict of interest.

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